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Cytotoxicity of Selected Pyridinium Oximes in Human SH-SY5Y Neuroblastoma Cell Line

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Pyridinium oximes are pharmacologically important nucleophilic agents acting as effective antidotes against poisoning by organophosphorus compounds that inhibit acetylcholinesterase (AChE; EC 3.1.1.7.). In this study, the cytotoxicity of 1-phenacylpyridinium-4-aldoxime chloride (FEPA-4) was analyzed in human SH-SY5Y neuroblastoma cell line highly expressing acetylcholinesterase. The concentrations of 0.5, 1, 2 and 4 mmol dm⁻³ and time-dependent effects at 1, 3, 6, 12 and 24 hours were tested in comparison with non-treated cells. In addition, neuroblastoma cells were treated with a well known antidote 1,1'-bis(pyridinium-4-aldoxime)trimethylene dibromide (TMB-4) at the concentration of 0.8 mmol dm⁻³, the highest studied concentration which in several non-neural cellular models induced no cytotoxicity. Cytotoxic effects *i.e.* altered cellular morphology and decreased cellular volume density quantified by stereological method were observed in FEPA-4 treated cells, while no cytotoxic effect was observed for 0.8 mmol dm⁻³ TMB-4. Possible mechanisms of observed FEPA-4 cytotoxicity in neuroblastoma cells are discussed.

Keywords
aldoximes
in vitro cytotoxicity
morphological analysis

INTRODUCTION

Many oximes and their metal complexes have shown significant and versatile bioactivity. Their biological functions and metabolizing mechanisms in living systems are usually related to their structure and chelating ability. Pyridinium oximes are best known as pharmacologically important nucleophilic agents that are effective antidotes against poisoning by organophosphorus compounds (pesticides, chemical warfare nerve agents, drugs used in

the treatment of cholinergic disorders).¹ Organophosphorus compounds inhibit acetylcholinesterase (AChE; EC 3.1.1.7.) which physiological role is hydrolysis of the neurotransmitter acetylcholine in the nervous system. Bispyridinium oximes, such as 1,1'-bis(pyridinium-4-aldoxime)trimethylene dibromide (TMB-4, shown in Figure 1), are the most potent antidotal agents. Even certain pyridinium derivatives that lack the oxime group, such as 1-phenacylpyridinium chloride and its 2-methylpyri-

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dinium derivative, act as protectors of AChE due to their capability to reversibly inhibit this enzyme.² Apparently they have antidotal efficacy in soman poisoning as well, ascribed to the reactivation or protection of AChE.³ 1-phenacylpyridinium-4-aldoxime chloride (FEPA-4, shown in Figure 1) serves as potential reactivator of the inhibited AChE although its antidotal properties are not known so far. FEPA-4 is known to react with aquapentacyanoferrate(II) by forming a well characterized substituted pentacyanoferrate(II) complex.^{4,5,6} Although the derivatives of 1-phenacylpyridinium aldoximes could exert a very complex action on the cholinergic and adrenergic nervous system and a strong anticholinesterase effect, they have been poorly studied as cytotoxic agents. Moreover, there have been no literature data on FEPA-4 bioactivity testing in *in vitro* and *in vivo* models. The aim of this study was to evaluate the cytotoxic and antiproliferative effects of FEPA-4 and TMB-4 in SH-SY5Y human neuroblastoma cell line, characterized by high expression of acetylcholinesterase.⁷

EXPERIMENTAL

Chemicals

FEPA-4 and TMB-4 were previously synthesized according to known procedures.^{4,8} Aqueous solutions of these compounds remain stable for days.

Cell Line

SH-SY5Y neuroblastoma cells were grown in culture flasks and cultivated in MEM medium supplemented with 10 % fetal bovine serum, L-glutamine (200 mmol dm⁻³), and antibiotics (penicillin and streptomycin, 100 µg cm⁻³).

FEPA-4 and TMB-4 Treatment of SH-SY5Y Cells

Neuroblastoma cells were seeded in 4 well plates 12 hours prior to addition of FEPA-4 and TMB-4. Cells were then incubated in duplicates in 0.5, 1, 2 and 4 mmol dm⁻³ FEPA-4 in serum-free medium, and in 0.8 mmol dm⁻³ TMB-4 in serum-free medium for 1, 3, 6, 12 and 24 hours. In parallel, non-treated cells were grown in a serum-free medium during the same experimental periods.

Morphological (Stereological) Analysis of Cytotoxic Effects

Changes in cellular morphology and cell viability were analyzed in native and hematoxylin-eosin stained FEPA-4 and TMB-4 treated *vs.* non-treated cell populations, by invert light microscopy (Zeiss Axiovert 200 microscope equipped with digital camera AxioCam MRc5). For a stereological analysis, a non-biased Weibel's 42-point multipurpose test system⁹ was applied at a magnification of 400x. The length of the test lines was 0.504 mm, whereas the test surface area was 0.02094 mm² for each analyzed microscopic field.^{9,10} The 4-well culture dish was placed under the microscope.

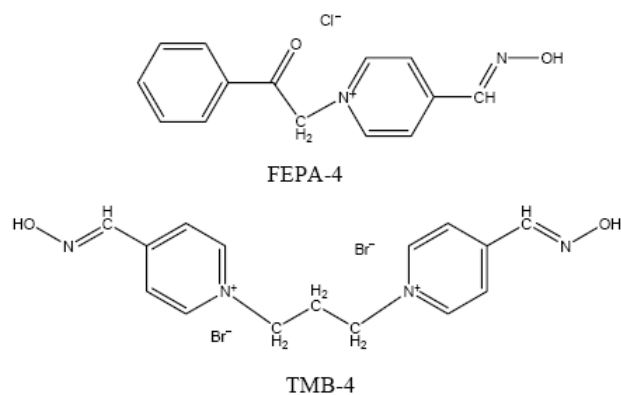


Figure 1. Structures of the tested aldoximes.

Each well had a surface area of 1 cm². The cells grown in the culture were focused (without refocusing). In order to determine the volume of cell clusters (volume density, Vv_c) that occupied a well in various cell cultures/protocols, a point counting method has been used. To determine Vv_c the following formula was used:^{9,10,11}

$$Vv_c = P_c / P_t \quad (1)$$

where P_c is number of hits on cultured cells whereas P_t is a number of test points ($P_t = 42$). A pilot stereological measurement of Vv_c has been made in order to determine the number of microscopic fields (n) needed for a statistically reliable data assessment.¹² The pilot measurement has been carried out on 20 microscopic fields. After this preliminary measurement, de Hoff's formula¹² was applied:

$$n = (20 \cdot s/x)^2 \quad (2)$$

where x is the mean value of Vv_c and s is the standard deviation. In our case, the number of microscopic fields to be assessed for each culture dish was 50. The final cellular volume densities were expressed as mean values $\pm s$ of data obtained in two independent experiments in which the counting of 50 microscopic fields was made in duplicate.

RESULTS AND DISCUSSION

According to our knowledge this is the first study dealing with *in vitro* effects of 1-phenacylpyridinium-4-aldoxime chloride (FEPA-4). Since structural characteristics of FEPA-4 indicate its antidotal activity through reversible inhibition and potential reactivation of acetylcholinesterase, a neuroblastoma cellular model highly expressing acetylcholinesterase was chosen.⁷ Results showed both concentration- and time-dependent effects of FEPA-4 on human SH-SY5Y neuroblastoma cells in comparison with non-treated cells. In order to estimate a range of concentrations exerting cytotoxic effects, cells were first treated with 1, 2 and 4 mmol dm⁻³ FEPA-4 during 24 hours. As shown in Figure 2, concentrations of 2 and 4

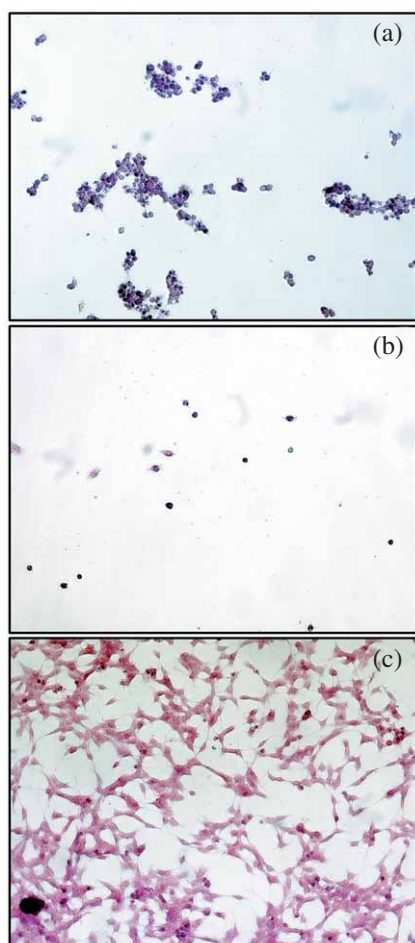


Figure 2. Morphology of SH-SY5Y cells treated with (a) 2 mmol dm⁻³ and (b) 4 mmol dm⁻³ FEPA-4 after 24 hours, in comparison with (c) non-treated cells (hematoxylin-eosin staining, magnification 200x).

mmol dm⁻³ FEPA-4 were found to be extremely cytotoxic for neuroblastoma cells, *i.e.* hematoxylin-eosin staining revealed that majority of cells were dead after 24 hours of treatment, with rounded cell bodies containing only nuclei surrounded by cytoplasmic remnants. Morphological changes (alterations) were quantified by stereological analysis,

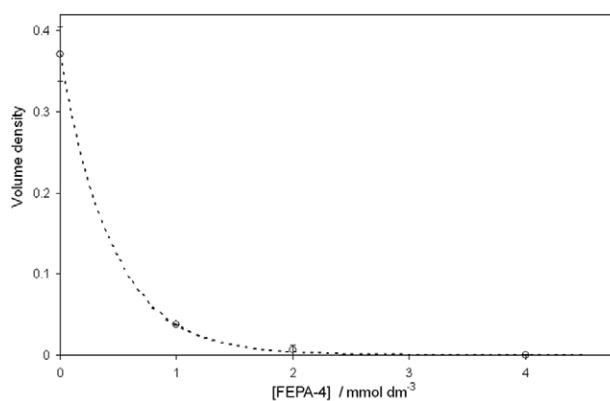


Figure 3. Cytotoxic concentration-dependent effects of FEPA-4 on SH-SY5Y neuroblastoma cells, quantified by stereological analysis. Data on cellular volume densities are expressed as mean values \pm SD.

which confirmed a dramatic decrease of cellular volume density as a function of a FEPA-4 concentration increase from 0.5–4.0 mmol dm⁻³ (Figure 3). Concentrations of 0.5 and 1.0 mmol dm⁻³ FEPA-4, showing less dramatic cytotoxicity, were further used for testing time-dependent effects of the compound during 1, 3, 6, 12 and 24 hours. Figure 4 shows that 0.5 mmol dm⁻³ FEPA-4 did not influence cellular proliferation ability; however slight alterations in cellular morphology appeared after 12 hours of incubation. At a concentration of 1.0 mmol dm⁻³ FEPA-4, inhibiting effects to cellular proliferation and morphological aberrations (rounded cell bodies, exudation of cytoplasmic elements, cell fragmentation) were first observed after 3 hours of incubation and proportionally more after 6, 12 and 24 hours (Figure 4). Quantification of the observed time-dependent morphological changes, expressed as cellular volume density changes in treated *vs.* non-treated cells, is shown in Figure 5. A concentration of 0.5 mmol dm⁻³ FEPA-4 induced a 30 % decrease in cellular volume density up to 12 hours of treatment. However, a slight continuous increase was observed in a later time period up to 24 hours, almost approaching the

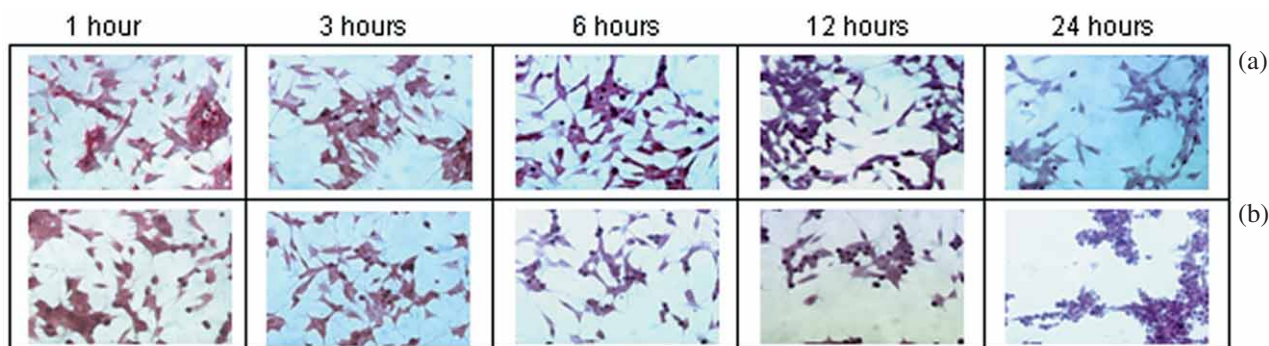


Figure 4. Morphology of SH-SY5Y cells treated with (a) 0.5 mmol dm⁻³ and (b) 1 mmol dm⁻³ FEPA-4 during 1, 3, 6, 12 and 24 hours (hematoxylin-eosin staining, magnification 200x).

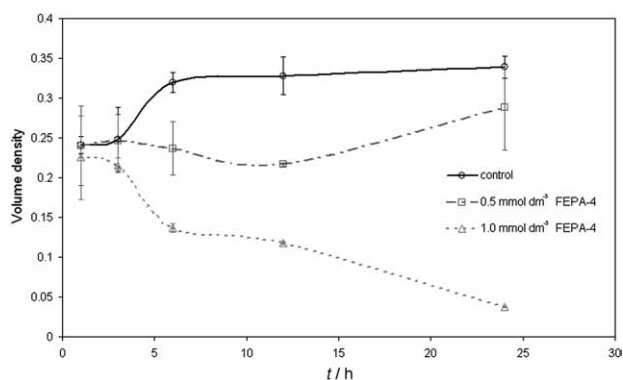


Figure 5. Time-dependent effects of FEPA-4 on SH-SY5Y neuroblastoma cells, quantified by stereological analysis. Data on cellular volume densities are expressed as mean values \pm SD.

control value of cellular volume density. It seems that after initial inhibition of cell proliferation induced by 0.5 mmol dm⁻³ FEPA-4, a certain fraction of FEPA-4-resistant cells recovered their proliferative ability. The observed sensitivity/resistance of neuroblastoma cells to FEPA-4 could be in relation to a cell cycle stage. On the other hand, incubation of cells with 1 mmol dm⁻³ FEPA-4 resulted in a continuous decrease of cellular volume density starting from 14 % decrease evidenced after 3 hours, 60 % decrease after 6 hours, up to 88 % decrease after 24 hours of treatment. Mechanism of FEPA-4 cytotoxicity in neuroblastoma cells and possible involvement of FEPA-4 in modulation of acetylcholinesterase activity *in vitro* will be further explored. Unlike similar pyridinium aldoximes, such as benzoylethylpyridinium-4-aldoxime chloride (BEPA-4, a higher homologue of FEPA-4) which has been tested *in vitro* and *in vivo*,³ in our studies of their physico-chemical properties, FEPA-4 was found to be well soluble in water and its solutions stable in physiological pH range (to be published elsewhere). Such advantageous properties make FEPA-4 a superior candida-

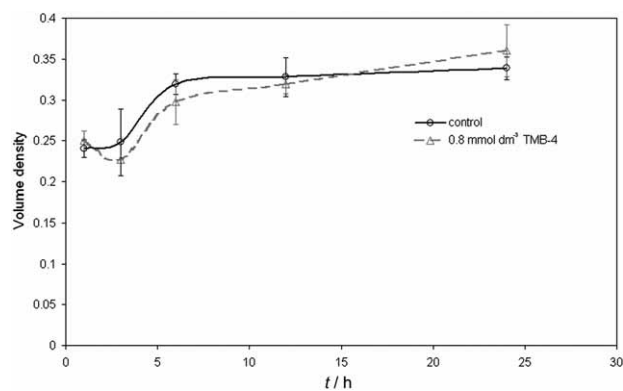


Figure 7. Time-dependent effects of TMB-4 on SH-SY5Y neuroblastoma cells, quantified by stereological analysis. Data on cellular volume densities are expressed as mean values \pm SD.

te for further *in vitro* and *in vivo* studies of bioactivity of such compounds.

Effects of a well known antidote 1,1'-bis(pyridinium-4-aldoxime)trimethylene dibromide (TMB-4) have been extensively studied *in vitro* and *in vivo*.¹ However, so far performed *in vitro* studies dealt only with various transformed non-neural cell lines. In this study, TMB-4 was tested in SH-SY5Y neuroblastoma cell line, proven to be a sensitive model for *in vitro* investigation of different antiproliferative/cytotoxic drugs¹³ as well as for studies of *in vitro* toxicity of organophosphorus compounds.¹⁴ SH-SY5Y cells were incubated in 0.8 mmol dm⁻³ TMB-4 during 1, 3, 6, 12 and 24 hours. This TMB-4 concentration, as the highest tested by other authors, was reported still not to be cytotoxic in non-neural cellular models during shorter time period (up to 4 hours).¹⁵ Figure 6 shows morphology of SH-SY5Y cells treated with TMB-4. Interestingly, starting with 3 up to 6 hours treatment a growth and elongation of neuritic extensions was observed indicating moderate cellular differentiation. However, in later periods no antiproliferative/cytotoxic effects were evidenced as confirmed by stereolo-

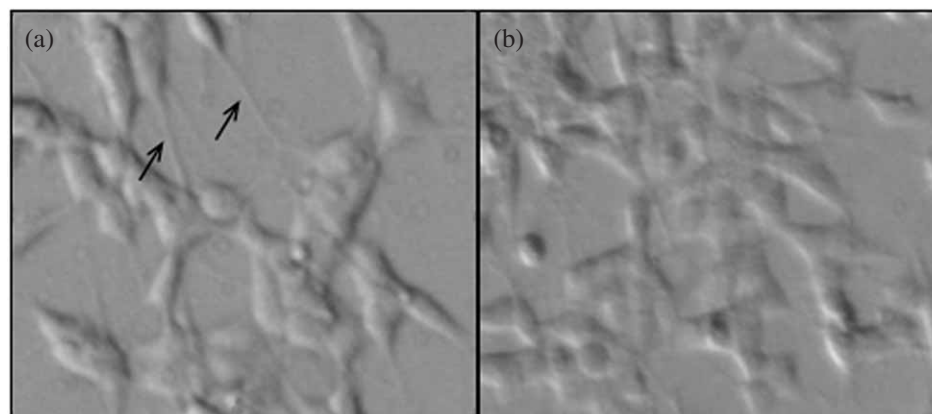


Figure 6. Morphology of (a) SH-SY5Y neuroblastoma cells treated for 6 hours with 0.8 mmol dm⁻³ TMB-4 (cell differentiation toward neuronal phenotype pointed by arrows), in comparison with (b) non-treated cells grown during the same time interval (native cells, magnification 140 \times).

gical quantification showing no difference in cellular volume densities between treated and non-treated cells (Figure 7). Higher doses of TMB-4 will be further tested in this neuronal cell model in order to determine its cytotoxic effects.

CONCLUSIONS

The study shows concentration- and time-dependent cytotoxic effects of FEPA-4 on human SH-SY5Y neuroblastoma cell line. However, the exact mechanism of observed FEPA-4 toxicity and possible involvement of FEPA-4 action to acetylcholinesterase is being further explored in this cellular model. No antiproliferative/cytotoxic effects of 0.8 mmol dm⁻³ TMB-4 were evidenced in SH-SY5Y neuroblastoma cell line.

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SAŽETAK

Citotoksičnost odabranih piridinijevih oksima u SH-SY5Y staničnoj liniji humanog neuroblastoma

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Piridinijevi oksimi, farmakološki značajni nukleofilni reagensi, učinkoviti su antidoti kod trovanja organofosforinim spojevima koji inhibiraju acetilkolinesterazu (AChE; EC 3.1.1.7.). U ovom je radu ispitana citotoksičnost 1-fenacilpiridinij-4-aldoksim klorida (FEPA-4) u ljudskoj neuroblastomskoj staničnoj liniji SH-SY5Y koja eksprimira acetilkolinesterazu. Ispitani su učinci u ovisnosti o koncentraciji (0,5, 1, 2 i 4 mmol dm⁻³) i vremenu (1, 3, 6, 12 i 24 sata), u usporedbi s kontrolnim stanicama. Također, stanice su tretirane poznatim antidotom 1,1'-bis(piridinij-4-aldoksim)trimetilen dibromidom (TMB-4) u koncentraciji od 0,8 mmol dm⁻³, najvišoj testiranoj koncentraciji koja nije izazvala citotoksičnost u nekoliko neneuralnih staničnih modela. U stanicama tretiranima s FEPA-4 uočeni su citotoksični učinci – poremećena stanična morfologija i smanjena volumna gustoća stanica utvrđena stereološkom metodom. Citotoksični učinci nisu uočeni u stanicama tretiranima s 0,8 mmol dm⁻³ TMB-4. Raspravljeni su mogući mehanizmi opažene citotoksičnosti FEPA-4 u neuroblastomskoj staničnoj liniji.